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Comparison of Hepatitis B Virus DNA Extractions from Serum by the QIAamp Blood Kit, GeneReleaser, and the Phenol-Chloroform Method

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The abilities of GeneReleaser and QIAamp to extract the hepatitis B virus (HBV) DNA template from serum for amplification by PCR were evaluated and compared with that of the standard phenol-chloroform method. Differences in the sensitivities of the three methods were revealed by nested PCR of HBV DNA extracted from serially diluted hepatitis B e antigen (HBeAg)-positive (high-titer) serum. Phenol-chloroform was found to be the most sensitive extraction method but was time-consuming and labor intensive, and the many steps required increased the possibility of contamination. In a titration of HBeAg-negative (low-titer) serum, all three methods coupled with nested PCR were capable of detecting low levels of HBV DNA. In the case of QIAamp and GeneReleaser, the extraction was relatively simple and rapid. The higher quantity of serum (200 μl) used in the QIAamp extraction did not provide higher sensitivity, possibly because of incomplete removal of *Taq* polymerase inhibitors from the serum or inadequate disruption of the virion. GeneReleaser was more efficient because it gave the same detection limit in low-titer serum as phenol-chloroform even though it utilizes only 5 μl of serum. However, it did not produce consistent amplifications of HBV DNA, giving false-negative results in 7 of the 50 cases (14%) in one experiment. Use of a larger volume of serum and replicate extractions may overcome this problem. Advantages thus exist in each of the extraction methods, and these should be weighed against the disadvantages when deciding which extraction method is appropriate.

The presence of hepatitis B virus (HBV) DNA in serum is a reliable marker of viral replication and infectivity, and PCR is becoming the preferred method for its detection (9). PCR is an extremely sensitive technique, which can detect as few as 10 HBV genomes per ml (1, 6). However, the efficacy of PCR amplification may be affected by the presence of a number of inhibitors of *Taq* polymerase in the serum, and for this reason thorough DNA extraction is required before amplification (10). Extraction of HBV DNA by incubation of serum with proteinase K in the presence of sodium dodecyl sulfate and subsequent phenol-chloroform extraction and ethanol precipitation are a standard technique for the isolation of HBV template DNA (5). This method is complicated, expensive, time-consuming, and unsuitable for treating high numbers of samples, and this hampers its routine use. For this reason, a number of quicker methods of isolating DNA have been developed (3, 5, 10, 14, 15) and a number of commercial DNA extraction kits have been introduced. This study compares the GeneReleaser and QIAamp kits to the standard phenol-chloroform extraction method.

MATERIALS AND METHODS

Samples. Hepatitis B surface antigen (HBsAg)-positive serum samples were obtained from 51 southern African blacks and were stored at −70°C. Forty-one were HBV carriers (HBsAg positive for more than 6 months), and 10 had hepatocellular carcinoma. Of the carriers, 21 were hepatitis B e antigen (HBeAg) positive, and the remaining 20 were HBeAg negative. All of the patients with hepatocellular carcinoma were HBeAg negative. Serum samples from healthy southern African black patients lacking serological markers for HBV served as negative controls. Commercially available kits (Abbott Laboratories, Chicago, Ill.) were used to detect HBV antigens.

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DNA extraction. (i) Phenol-chloroform extraction. For phenol-chloroform DNA extraction, a 125-μl aliquot of serum was incubated at 70°C for 2 h in the presence of 400 mg of proteinase K ml^{-1}, 1% sodium dodecyl sulfate, 2.5 mM disodium EDTA, and 25 mM sodium acetate. The suspension was sequentially extracted with phenol and then chloroform. The DNA was precipitated with 0.3 M sodium acetate and then with absolute ethanol, washed with 70% ethanol, vacuum dried, and then dissolved in 50 μl of best-quality water (BQW).

(ii) GeneReleaser. GeneReleaser is a proprietary reagent that can release DNA from serum. Lysis is accomplished directly in the amplification tube in a thermocycler. A 20-μl volume of GeneReleaser (Bioventures Inc., Murfyboro, Tenn.) was added to 5 μl of serum, and the extraction was performed in a programmable thermal cycler (Perkin-Elmer, Norwalk, Conn.) according to the manufacturer's directions as follows: 65°C held for 30 s, 8°C held for 30 s, 65°C held for 90 s, 97°C held for 180 s, 8°C held for 60 s, 65°C held for 180 s, 97°C held for 60 s, 65°C held for 60 s, and 80°C held until the PCR mixture was added. The end product (25 μl) was used as the template for the 100-μl first-round PCR mixture.

(iii) QIAamp blood kit. The QIAamp blood kit (Qiagen, Inc., Hilden, Germany), another method for nucleic acid purification and removal of amplification inhibitors, was used according to the manufacturer's instructions. A 200-μl aliquot of serum was incubated with QIAGEN protease and buffer AL at 70°C for 10 min. Potentially infectious agents were inactivated by incubation at 95°C for 15 min. The lysate was applied to a QIAamp spin column, spun, and washed three times with buffer AW and finally eluted with 50 μl of BQW.

(iv) Controls. Sera positive for HBsAg, HBeAg, and HBV DNA (by slot blot hybridization) were used as positive controls. BQW and serum samples from healthy blacks lacking all HBV serological markers were used as negative controls. To test the relative sensitivities of the three procedures, HBV DNA was isolated from HBV-positive sera serially diluted in HBV-negative control serum. One high-titer (HBeAg-positive) serum sample and one low-titer (HBeAg-negative) serum sample were serially diluted.

PCR. (i) Primers. Primers specific for HBV core gene sequences were used for single-round PCR, whereas primers specific for the precore-core region were used for nested PCR (Table 1).

(ii) Amplification. The final PCR mixture contained 0.02 U of DynaZyme DNA polymerase μl^{-1} (version 2.0; Finnzymes OY, Espoo, Finland), 200 μM each deoxynucleoside triphosphate, 1 μM each primer, 50 mM KCl, 10 mM Tris HCl (pH 8.8), 1.5 mM $MgCl^2$, and 0.1% Triton X-100.

For phenol-chloroform and QIAamp DNA extracts, 2.5 μl of target DNA was added to 22.5 μl of the appropriate master mixture. The volume of GeneReleaser used to accomplish cell lysis was compensated by deducting the equivalent volume of BQW from the components of the amplification reagents to maintain

TABLE 1. Oligonucleotide primers used in the study

Primer[a]	Sequence	Position[b]	Size (bp)
Single-round PCR			
2269 (+)	5′ GGA GTG TGG ATT CGC ACT	2269–2286	147
2415R (−)	5′ TGA GAT CTT CTG CGA CGC	2415–2398	
Nested PCR			
Inner primers			
1730 (+)	5′ CTG GGA GGA GTT GGG GGA	1730–1747	314
2043R (−)	5′ CA ATG CTC AGG AGA CTC TAA CGG	2043–2021	
Outer primers			
1763 (+)	5′ GGT CTT TGT ACT AGG AGG CTG	1763–1783	204
1966R (−)	5′ GTC AGA AGG CAA AAA CGA GAG	1966–1946	

[a] +, sense; −, antisense.
[b] Nucleotide positions of HBV *ayw* (GenBank accession no. V00866), with the *Eco*RI cleavage site at position 1 (4).

the appropriate concentrations in the final reaction volume. Thus, 75 μl of this master mixture was added to 25 μl of GeneReleaser extract.

The first-round PCR was performed in a programmable thermal cycler (Perkin-Elmer) with the following three-step cycling profile: 94°C for 30 s (denaturation), 62°C for 50 s (annealing), and 72°C for 50 s (polymerization) for a total of 40 cycles, after which came a final extension of 10 min at 72°C.

For the second-round PCR, a 2.5-μl aliquot of the first-round amplification product was reamplified with internal primers as described above, except that the annealing temperature was decreased to 58°C. For single-round PCR, annealing was carried out at 51°C with all other steps as described above.

All amplifications were performed with HBV-positive and HBV-negative controls. PCR controls to detect contamination consisted of BQW added to the PCR mixture instead of DNA; a water control was interspersed between each serum sample. To avoid cross-contamination and false positive results, the precautions and procedures suggested by Kwok and Higuchi (7) were strictly adhered to. DNA extraction, PCR, and electrophoresis were performed in physically separated venues.

(iii) Detection of amplified product. A 20-μl aliquot of the amplified product was electrophoresed on an 2% agarose gel. Bands of the appropriate size (147 bp for single PCR or 204 bp for nested PCR) were visualized under UV light after ethidium bromide staining. Each sample was analyzed in duplicate and read as positive or negative only when the two determinations agreed. Positive and negative controls were run in parallel with every assay.

The specificity of the amplified band was confirmed by Southern blot hybridization with ^{32}P-labelled HBV DNA probe (HBV DNA in the pBV325 vector [*adr* subtype], which was purified and supplied by C. Brechot, Institut National de la Santé et de la Recherche Médicale U 370, Institut Pasteur, Paris, France).

RESULTS AND DISCUSSION

To optimize the detection of HBV DNA in serum, a pilot experiment was carried out comparing single-round PCR versus nested PCR of phenol-chloroform DNA extracts. As shown in Table 2, HBV DNA could be detected in all HBeAg-positive subjects by using either single-round or nested PCR. Serum samples of patients of this sort would be expected to have more than 10^6 particles per ml (2). However, single-round PCR was less sensitive in detecting HBV DNA present in the sera of HBeAg-negative subjects. This too would be expected, since seroconversion from HBeAg to anti-HBe can be accompanied by a decrease in virion concentration to between 10^2 and 10^6 per ml (2). Thus, the sensitivity of the method can be increased by the use of nested primers in a double round of PCR, as previously reported by Kaneko et al. (5). However, the sensitivity of the nested PCR is also its potential drawback, because it increases the chances of inadvertent contamination causing false-positive results. Therefore, extreme caution was exercised to avoid cross-contamination (7).

TABLE 2. Detection of HBV DNA in phenol-chloroform extracts

Serological status	Single PCR[a] (%)	Nested PCR[b]	P[c]
HBeAg positive	7/7 (100)	21/21 (100)	
HBeAg negative	5/22 (23)	29/30 (97)	0.007

[a] Primers 2269 (+) and 2415R (−) in the core region.
[b] Outer primers 1730 (+) and 2043R (−) in the precore and core region; inner primers 1763 (+) and 1964R (−) in the precore and core region.
[c] Chi-square testing.

In the present study, when the three different methods of HBV DNA extraction were compared, nested PCR was used. DNA extracted with either phenol-chloroform or the QIAamp blood kit provided equally adequate templates for PCR, with all 50 samples giving positive amplification independently of the HBeAg status of the samples. On the other hand, GeneReleaser gave false-negative results in 7 of the 50 specimens (14%) in a single experiment. In a second amplification attempt following reextraction, these samples were positive for HBV DNA. Since six of these specimens were from HBeAg-negative subjects, the false-negative results are most likely due to a statistical error of sampling (12) because of the very small volume of serum used and/or small number of HBV particles in the sample. Saiki et al. (11) also reported that limiting dilutions of DNA template do not produce consistent amplifications. Alternatively, it is possible that *Taq* polymerase inhibitors in the serum (14) were not adequately removed, diluted, or inactivated during the pre-PCR DNA extraction procedure by GeneReleaser, thus causing the false-negative results. However, this is an unlikely explanation, because positive controls amplified consistently and the temperatures used in the GeneReleaser method should reliably inactivate inhibitory serum factors (3).

To evaluate the relative efficiency of each extraction procedure and its influence on the extent of amplification, HBV-containing samples were serially diluted in HBV-negative serum prior to extraction. Both high-titer (HBeAg-positive) and low-titer (HBeAg-negative) sera were used. Table 3 shows the detection limit (the highest dilution at which HBV DNA was still detectable by PCR) of each extraction method.

As shown in Table 3, HBV DNA from an HBeAg-positive (high-titer) sample could be detected at a dilution of 10^{-12} following phenol-chloroform extraction. On the other hand, the phenol-chloroform method was not found to be more efficient than the other two methods in the extraction of HBV DNA from HBeAg-negative serum. This may result from the observation that in the low-titer serum, the limiting factor in the sensitivity of detection is the low initial viral concentration (8). The fact that samples from the first round of amplification were negative for HBV DNA regardless of the method of extraction (Table 3) supports this belief. Furthermore, it is possible that losses of sample at the organic-aqueous inter-

TABLE 3. Results of the sensitivity assay of HBV DNA detection by PCR in high- and low-titer serum samples with different DNA extraction procedures

HBeAg status	DNA extraction method	Detection limit after[a]	
		PCR I[b]	PCR II[c]
+ (high titer)	Phenol-chloroform	10^{-3}	10^{-12}
	QIAamp	10^{-3}	10^{-6}
	GeneReleaser	10^{-1}	10^{-3}
− (low titer)	Phenol-chloroform	—[d]	10^{-1}
	QIAamp	—	10^{0}
	GeneReleaser	—	10^{-1}

[a] Detection limit, the highest dilution of HBV-containing serum in HBV-negative serum at which HBV DNA was detectable by PCR and agarose gel electrophoresis.
[b] I, first-round PCR [primers 1730 (+) and 2043R (−)].
[c] II, nested PCR [outer primers 1730 (+) and 2043R (−); inner primers 1763 (+) and 1966R (−)].
[d] —, HBV DNA was not detected.

phase during extraction and ethanol precipitation (5) are more significant in low-titer serum than in high-titer serum, resulting in decreased extraction efficiency. The phenol-chloroform extraction method is complicated and time-consuming and requires extensive use of disposable plasticware, and, in agreement with others (3), we found that cross-contamination occurred more frequently with this extraction method than with any of the other two methods.

Although the 200-μl volume of serum used in the QIAamp extraction method versus the 125-μl volume used in the phenol-chloroform method theoretically contained approximately double the amount of target DNA, it did not provide higher sensitivity (Table 3). These results are probably due to the incomplete removal of inhibitors of *Taq* polymerase found in the serum (13) or inadequate disruption of the virions. However, the QIAamp extraction method is less time-consuming and requires less plasticware than the phenol-chloroform method.

Although the extraction of HBV DNA with GeneReleaser is very efficient and gives the same detection limit in low-titer samples as phenol-chloroform (Table 3) (even though it utilizes a small volume of serum), its major disadvantage is false-negative results. Moreover, because GeneReleaser extraction and first-round PCR are performed in a single tube, each extraction can be used with only one set of outer primers in a 100-μl volume, whereas in both of the other methods as many as five sets of primers can be used after a single extraction. However, GeneReleaser is the most economical, time-effective method, requiring a small amount of serum, and the minimum handling of samples greatly reduces the chance of contamination.

Advantages thus exist in using each of the extraction methods, and these should be weighed against the disadvantages when deciding which extraction method is appropriate for the circumstances of the investigation.

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